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Infection conditions of *Fusarium graminearum* in barley are variety specific and different from those in wheat

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Bertossa, Mario ; Keller, Beat ; Vogelgsang, Susanne

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Infection conditions of *Fusarium graminearum* in barley are variety specific and different from those in wheat

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system. An up to three times higher DON contamination in the 15 °C treatments for the feed barley variety Ascona was detected compared with the 10 °C and 20 °C treatments. For the malting barley variety Concerto, the prolonged humidity durations had a stronger effect under all tested temperatures and resulted in up to two times higher DON contaminations. In addition, field experiments where spore deposition during anthesis as well as disease incidence, fungal amount and mycotoxins were observed, showed that the overwintered straw treatment resulted, depending on the year, in a three times higher FG incidence and DON content compared with the control and freshly inoculated straw treatment.

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Keywords *Fusarium graminearum* · Barley · Epidemiology · Forecasting · Spore deposition · Weather conditions

Introduction

Fungi of the genus *Fusarium* can cause *Fusarium* head blight (FHB), one of the most noxious cereal diseases. In Switzerland, *F. graminearum* (FG) is the most occurring species in barley and wheat (Schöneberg et al. 2016; Vogelgsang et al. 2011) and the typical symptoms are discoloured and shrivelled grains (Parry et al. 1995). Apart from reduced grain quality and yield, FG produces mycotoxins such as deoxynivalenol (DON) (Bottalico and Perrone 2002; Osborne and Stein 2007). Mycotoxins

are harmful to humans and animals and thus the European Commission has set a maximum level for DON in unprocessed barley intended for human consumption of $1250 \mu\text{g kg}^{-1}$ (The European Commission 2006), which was adopted in the respective Swiss legislation.

In Switzerland, barley is mainly grown as feed but also used for human consumption as malt and beer or for baking and cooking purposes. During beer processing, the mycotoxin content decreases but still low amounts can remain in the final product (Habler et al. 2017; Inoue et al. 2013). Feed barley is generally used directly on farm and thus not regularly analysed compared with cereals used for human consumption which can result in undetected, elevated mycotoxin concentrations. Pigs fed with DON contaminated barley showed a reduced feed intake, diarrhoea and vomiting (reviewed in D'Mello et al. 1999).

Knowledge about the most influencing cultivation measures such as crop rotation, choice of the variety and tillage is important to reduce the risk of a FG infection in barley and was assessed during the recent years (Schöneberg et al. 2016). In addition, data about the most susceptible growth stages and favourable climatic conditions, which were shown to affect the infection with FG, are necessary to predict the occurrence in a particular field (Cowger et al. 2009; Brustolin et al. 2013). Since FHB epidemics are strongly dependent on the effective weather conditions, a control with fungicides is not always required (McMullen et al. 1997; Brustolin et al. 2013). Hence, a forecasting system can avoid unnecessary fungicide applications (Wegulo et al. 2015). Temperature and precipitation are embedded in several forecasting systems such as FusaProg in Switzerland (Musa et al. 2007), DONCast in Canada (Schaafsma and Hooker 2007) and Qualimètre® in France and Belgium (Froment et al. 2011). However, they all focus on wheat or maize and until to date, no forecasting system for FHB in barley is available. Models on FHB incidence in barley were tested in China since the 1980s, although only for a particular cultivar and region (cited in Choo 2009).

Hence, the main objectives of our studies were: (1) to assess the most critical temperature and moisture conditions for an FG infection in barley under controlled climate chamber conditions, and (2) to observe the FG spore deposition during the anthesis period in relation to the prevailing weather conditions in the field.

Material and methods

Inoculum preparation

The inoculum preparation for the straw used in the field experiment was done as described in Martin et al. (2017) with the following modifications: colonies were grown on Potato Dextrose Agar (PDA) for one week at 12 h UV-light / 12 h dark at 18 °C. One hundred ml autoclaved V8-medium in a 250 ml Erlenmeyer flask were inoculated with two FG PDA discs ($\phi = 0.5 \text{ cm}$), closed with a sterile plug and shaken for seven days at 200 rpm at 22 °C. Afterwards the liquid media was filtered through kitchen roll and subsequently centrifuged. The supernatant was discarded and the occurring spore pellet was suspended in sterile water.

The inoculum preparation for the climate chamber experiments were done according to Vogelgsang et al. (2008) with the following modifications: inoculum production was executed on PDA and each plate was washed with 20 ml 0.0125% Tween® 20 (Sigma-Aldrich, Darmstadt, Germany) water.

Three FG strains, all isolated in 2013 from Switzerland and known to produce DON, were chosen. The geographic origin and host from which the used strains were derived are indicated in online resource S1. The isolates were stored as single conidia isolates in 50% glycerine water at -70 °C until use.

Climate chamber experiment

The two row spring barley varieties, Ascona and Concerto were used to study the influence of temperature and the duration at 99% relative humidity (RH). Ascona (Saatbau Linz, Austria), is used as feed, whereas Concerto (Limagrains, United Kingdom) is used for malting purposes. The uncoated seeds were placed into germination boxes lined with three wet filter papers ($150 \times 130 \text{ mm}$; Type 614, Macherey-Nagel, Düren, Germany) and incubated for 36 h at 5 °C in the dark. Subsequently, the boxes were incubated at 18 °C for 72 h in an incubator (RUMED, Rubarth Apparate GmbH, Laatzen, Germany) to ensure an equal development of the plants. Three germinated seedlings were planted in pots ($\phi 15 \text{ cm}$, 13 cm height) in standard soil (Oekohum GmbH, Herrenhof, Switzerland) at a depth of two to three cm. Climate chamber conditions were 18 °C / 75% RH during the day and 13 °C / 85% RH during the night for the first 14 days, then 20 °C / 70% RH (day) and

15 °C / 80% RH (night) until decimal code (DC) 83 (Zadoks et al. 1974). The conditions were then set to 22 °C / 70% RH (day) and 18 °C / 70% RH (night) until harvest. Each pot was fertilised from DC 41 until DC 83 with 250 ml of a 1% dilution Wuxal® P Profi (Manna, Düsseldorf, Germany).

At mid anthesis (DC 65), one spike per seedling, in total three spikes per pot, were spray inoculated using a pressure of 2 bar with 30 ml spore suspension (2×10^5 spores ml^{-1}) containing the three different FG strains (online resource S1) to mimic a population. Subsequently, the plants were transferred into a climate chamber without light, in which the combinations of temperature and humidity duration were tested: 10 °C / 4 h, 10 °C / 8 h, 10 °C / 12 h, 15 °C / 4 h, 15 °C / 8 h, 15 °C / 12 h, 20 °C / 4 h, 20 °C / 8 h and 20 °C / 12 h. A suspension using only 0.0125% Tween® 20 water at 20 °C / 12 h served as a spray inoculation control treatment. Immediately after the respective incubation treatments, the plants were transferred into a climate chamber with 20 °C / 70% RH (day) and 15 °C / 80% RH (night).

Set up of the field experiment

Location

The field experiments were conducted at the Agroscope research institute in Zurich, Switzerland (N 047° 24,430; E 008° 30,597) over two years, 2015 and 2016. The previous crop in both years was potato.

Straw inoculation, plot preparation and field management

The experiment consisted of a randomised complete block design with four replicate blocks. The treatments (plot size 3 m × 9 m) comprised three different straw inoculation methods: I: autoclaved straw (control), II: freshly inoculated straw and III: overwintered straw. For the “overwintered” treatment, barley straw from field experiments artificially inoculated during flowering with FG in the previous year was taken after harvest and stored at 5 °C in the dark until application.

For the control treatment, and the freshly inoculated treatment, barley straw harvested in 2014 was autoclaved for 30 min at 121 °C. For the treatment, “freshly inoculated”, the straw was inoculated in wooden boxes by stacking one layer of straw and subsequently spraying a FG spore suspension (2×10^5 spores ml^{-1})

containing the three different FG strains (online resource S1) before adding the next layer of straw. The same procedure was done for the control treatment, and the “overwintered straw” treatment using only Tween® 20 water. The applications were done using a backpack sprayer (Flox 10, Birchmeier, Stetten, Switzerland). Twenty four kilogram straw per treatment were sprayed with twelve litres of the spore suspension or Tween® 20 water. The wooden boxes were placed into climate chambers at 20 °C, 99% RH for two to three days. Once per day, the straw was mixed by hand, to ensure a homogenised infection. Before the straw application, subsamples of each treatment were placed on PDA to check the presence/absence of FG.

Seedbed preparation was done by ploughing (September 2014/2015) followed by a field cultivator treatment and subsequent harrowing (both October 2014/2015). Sowing of the six-row winter barley varieties Meridian and Semper (KWS Lochow GmbH, Germany) (seed density 0.3 t ha^{-1} , row distance 15.5 cm), was done in October 2014 and 2015, respectively. The fertilisation and plant protection applications in the respective years are indicated in online resource S2.

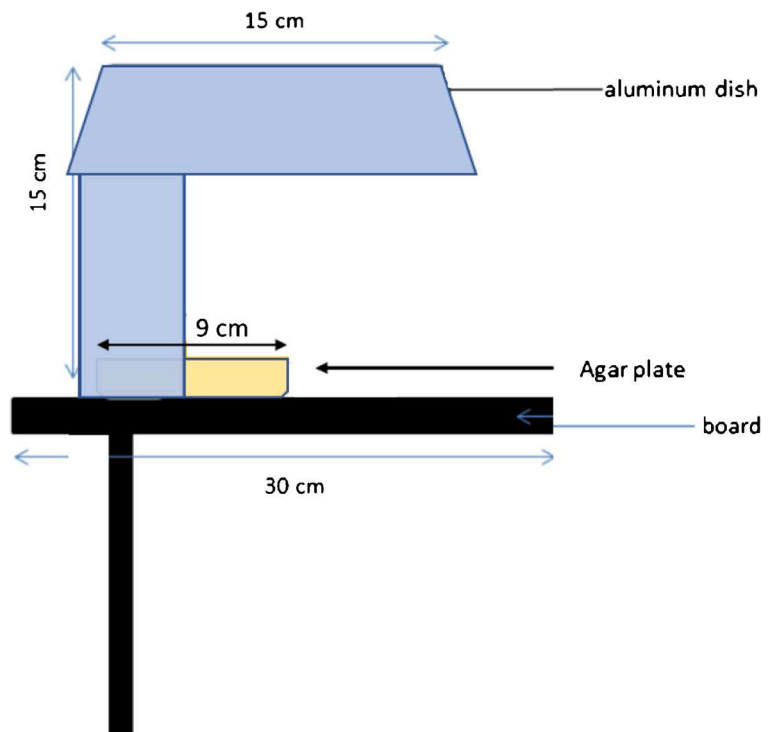
Adjacent at the length to the treatment plots where the straw was applied, a side plot of the same dimension with either Meridian or Semper (but without straw) was sown. The entire plot was additionally surrounded by triticale plots of 3 m × 12 m to minimise the risk of cross contamination (online resource S3). The straw was applied to the respective plots at DC 25 in 2015 and 2016, respectively. In each plot, 2.5 kg straw was applied which equals the average amount in a direct tillage field.

All plots were harvested with a plot combine harvester at the end of July 2015 and beginning of August 2016, respectively. Weather data (daily mean temperatures, RH and sum of precipitation) during the entire cropping season were obtained from a nearby weather station operated by the Federal Office of Meteorology and Climatology, MeteoSwiss.

Spore traps

Observation of the spore deposition during the anthesis period was done by the aid of spore traps as shown in Fig. 1. These consisted of an iron rod that was bent at a right angle at the upper end. At this angle, two holes were drilled and a wooden board about 30 cm long and 10 cm wide was screwed onto the iron rod. An aluminium dish (15 × 11 × 4.5 cm, type 150550, Company

Fig. 1 Schematic construction of the spore trap used in the field experiments



Pacovis AG, Stetten, Switzerland) was placed in a vertical position on the board. At the upper end of the aluminium dish, a second one of the same type was attached in a horizontal position. A Petri dish (\varnothing 9 cm) with a *Fusarium* selective agar medium containing pentachloronitrobenzene (PCNB) (Sigma-Aldrich®, Buchs, Switzerland), also known as Nash-Snyder medium (Papavizas 1967), was placed under this construction, and was thus protected from rain, direct sunlight and rapid drying out. In addition, two small nails were placed at a distance of nine cm from the vertical aluminium dish in order to fix the Petri dish onto the board.

The spore traps were placed on the height of the barley heads in northerly direction in order to protect the agar plates by the vertical aluminium dish from higher temperatures and irradiation at noon. For each plot, one spore trap was placed during the anthesis period and agar plates were exchanged every 24 h and subsequently incubated at 12 h UV / 12 h darkness at 18 °C for 6 days. To identify the different *Fusarium* species, colonies were transferred onto PDA and SNA (Speziell nährstoffarmer Agar (Leslie and Summerell 2006)) and incubated at the above mentioned conditions. After the incubation process, the different

Fusarium species were identified based on their morphology and according to Leslie and Summerell (2006).

Fusarium graminearum disease rating

The disease rating in the climate chamber experiment was done by counts of visibly infected spikelets per spike of the three inoculated spikes seven and 14 days past inoculation (dpi) and expressed in percent severity of the total number of spikelets per spike.

For the field experiment, the disease rating was performed twice by randomly selecting ten times ten ears of each plot. The first rating was performed once the first symptoms appeared (13 days after flowering (daf) at DC 73 in 2015, 18 daf (DC 73) in 2016) and the second rating followed after one more week (20 daf (DC 77) in 2015 and 25 daf (DC 77) in 2016). The total number of spikelets from 100 randomly selected ears were counted and averaged to calculate the severity in percent.

Fusarium species incidence

Incidences of *Fusarium* species in barley grains from the field experiment were determined with the seed

health test method described in Vogelgsang et al. (2008). Therefore, grains were surface-sterilised (10 min, 1% chloramine T) and 100 grains were placed on potato dextrose agar (10 grains plate⁻¹) and incubated and identified as described above.

Quantification of *Fusarium graminearum* by quantitative PCR

Quantitative PCR (qPCR) was performed to determine the amount of FG DNA in milled grain samples from the climate chamber experiment. The extraction was done using the NucleoSpin® 96 Plant II Kit (Macherey-Nagel, Düren, Germany) and measurement of the DNA concentration was done by measuring the emission of the fluorescent PicoGreen® (Promega, Madison, USA) as described in Schöneberg et al. (2018). Due to the high amounts of FG DNA, the extracted samples were diluted 1:200 to be within the range of the standard curve. All reactions were analysed in a CFX96™ Real-Time PCR Detection System–IVD (Bio-Rad, Hercules, USA) in a 96-well plate format (Hard-shell full-height 96-well semi-skirted PCR plates, BioRad, Hercules, USA). In every performed assay, all standards and the negative control (double distilled water) were run as triplicates.

The plasmid needed to establish the standard curve was prepared according to instructions of the Promega pGem®-T and pGem®-T Easy Vector Systems Kit (Promega, Madison, USA) with *E. coli* DH5α. Plasmid DNA from *E. coli* DH5α recombinants was extracted and purified with the QIAprep® Miniprep Kit (Qiagen Scientific, Venlo, The Netherlands). The used plasmid contained a 284 bp fragment (online resource S4) which is specific for FG (Nicholson et al. 1998). The standard curve was established using the diluted plasmid and barley matrix without FG DNA. The standard curve consisted of five standards which were prepared as a dilution series with a dilution factor of five in a range from 12,500 copies per reaction to 20 copies per reaction. The limit of determination (LOD) and the limit of quantification (LOQ) were determined from the dilution series of the standard curve. Therefore, the double amount of the lowest standard (20) was set as LOQ and the LOD was set as one tenth of the LOQ. Sample values below the LOD or LOQ were replaced by a constant value of LOD/2 or LOQ/2.

The used qPCR method for FG was originally developed by Brandfass and Karlovsky (2006) and adjusted by using a commercially available kit instead of

preparing it with the individual ingredients. The amplification mix consisted of 0.4 µl primer pairs (Microsynth AG, Balgach, Switzerland) Fg16N F (5' ACAGATGACAAGATTTCAGGCACA 3') and Fg16N R (5' TTCTTTGACATCTGTTCAACCCA 3') (Brandfass and Karlovsky 2006), 10 µl IQ SYBR® Green Supermix (Bio-Rad, Cressier, Switzerland) and 4.6 µl ddH₂O. The qPCR volume per well was 20 µl and contained 15 µl amplification mix and 5 µl diluted sample DNA. The initial melting curve was also adjusted and consisted of 3 min at 95 °C to activate the polymerase instead of 1.5 min, followed by subsequent 40 cycles instead of 35 cycles with the following conditions: 30 s at 92 °C instead of 94 °C, 45 s at 64 °C, 45 s at 72 °C. The final elongation was performed for 5 min at 72 °C.

Mycotoxin measurement in harvested grains

Deoxynivalenol was measured using a competitive Enzyme Linked Immunoabsorbent Assay (ELISA), Ridascreen® FAST DON (R-Biopharm AG, Darmstadt, Germany). For the field experiments, sample preparation and ELISA assay was conducted according to manufacturers' instructions. Because only the three inoculated ears per pot were harvested a reduced amount of milled samples as indicated by the manufacturer was available for the climate chamber experiment. Hence, the extraction procedure was modified as follows: A sample volume of 70 mg flour was weighted into a two ml Eppendorf tube® and 1.4 ml demineralised water was added. The samples were vortexed for 10 s and subsequently shaken on a horizontally shaker (Lab-Shaker Typ LSR-V, Birsfelden, Switzerland) at 250 rpm for 15 min at room temperature. Afterwards, the samples were centrifuged at 13.000 rpm for 5 min at room temperature in a table top centrifuge (Biofuge 13, Heraeus, Osterode am Harz, Germany). Three aliquots of each 350 µl supernatant were then pipetted into 1.2 ml collection microtubes (Qiagen, Hombrechtikon, Switzerland).

Samples absorbance were measured using a spectrometer (Tecan Sunrise™) and the toxin concentrations were estimated via a standard curve of known DON concentrations by Ridasoft Win 1.84 software (R-Biopharm AG). The limit of detection (LOD) was 222 µg kg⁻¹, individual values below the LOD were replaced by a constant value of 111 µg kg⁻¹.

Statistical analysis

For statistical analysis, the software R Version 3.3.2, R Studio Version 1.0.136 and the packages agricolae, lme4 and lsmeans were used (R Core Team 2015; R Studio Team 2015; de Mendiburu 2014; Lenth 2016; Bates et al. 2015). The package agricolae was used for the Tukey post-hoc test of the field experiments and the calculation of the correlations. The packages lme4 and lsmeans were used for analysis of the climate chamber experiments with a linear mixed effects model. Data from all experiments were verified graphically using plots of fitted values versus the root of the standardised residuals for homogeneity of variances and using normal Q-Q plots for normality of residuals. In addition, the Levene-test was used to verify homogeneity of variances and the Shapiro-Wilk test was used to check for normality of residuals. To meet the requirements for the post-hoc and correlation tests, DON and DNA data were log transformed, whereas untransformed data were used for the disease rating and seed health test data.

To investigate significant influencing factors, a linear mixed effects model was created. For the climate chamber experiment, the factors temperature and humidity duration were set as fixed factors, whereas experiment repetition and block were set as random factors. The analysis for the climate chamber experiments were made for each variety separately. Obtained significant factors were then examined using the Tukey method for pairwise comparison of least-square means ($\alpha = 0.05$). For the field experiments, an analysis of variance followed by Tukey's honest significant difference test ($\alpha = 0.05$) was used. The analysis for the field experiments were made for each variety and year separately, since the observed differences were not similar in both years.

Using the transformed data, Pearson correlation was calculated to determine the relationship between disease rating, qPCR and DON data for the climate chamber experiment and between disease rating, seed health test and DON data for the field experiment. For figures, the software SigmaPlot[®] Version 13.0.0.83 (Systat Software, Inc.) and Microsoft[®] Excel 2013 were used and untransformed data were plotted.

Results

Climate chamber experiment

The FG DNA amount and DON content in the milled grains of the inoculated plants was above the LOD in 94% and 98%, respectively. Neither DON nor FG DNA was measured in the control treatment grains. In general, the disease symptoms increased from the first to the second rating and revealed the same differences, hence, only results from the first date are presented here.

The lme revealed temperature and the interaction of temperature and humidity duration as significant factors on disease severity for the variety Ascona (Table 1). No significant differences between the humidity durations were observed within the three tested temperatures (Fig. 2). The inoculation treatments 10 °C / 12 h and 20 °C / 4 h showed significantly lower disease severity compared with the treatments 15 °C / 8 h ($p = 0.009$ and $p = 0.018$, respectively) and 15 °C / 12 h ($p = 0.025$ and $p = 0.046$, respectively). Also, the treatment 15 °C / 8 h revealed a higher disease severity and was significantly different from the 10 °C 8 h ($p = 0.033$) and the 20 °C 8 h ($p = 0.032$) treatments (Fig. 2).

Table 1 Summary of linear mixed effects model for variables predicting *Fusarium graminearum* (FG) disease severity after 7 days past inoculation (7dpi), FG DNA, and deoxynivalenol

Variable	Variety	Degrees of freedom	FG disease severity 7dpi		FG DNA		DON	
			F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)
Temperature	Ascona	2	10.206	<0.001	8.565	<0.001	4.221	0.021
Humidity duration	Ascona	2	1.412	0.254	2.893	0.066	0.048	0.953
Temperature * Humidity duration	Ascona	4	2.645	0.045	1.222	0.315	3.001	0.028
Temperature	Concerto	2	4.229	0.021	0.538	0.588	0.559	0.576
Humidity duration	Concerto	2	0.477	0.624	4.221	0.021	7.667	0.001
Temperature * Humidity duration	Concerto	4	0.659	0.624	1.628	0.185	0.341	0.849

(DON) content of the climate chamber experiments with the barley varieties Ascona and Concerto; $n = 108$

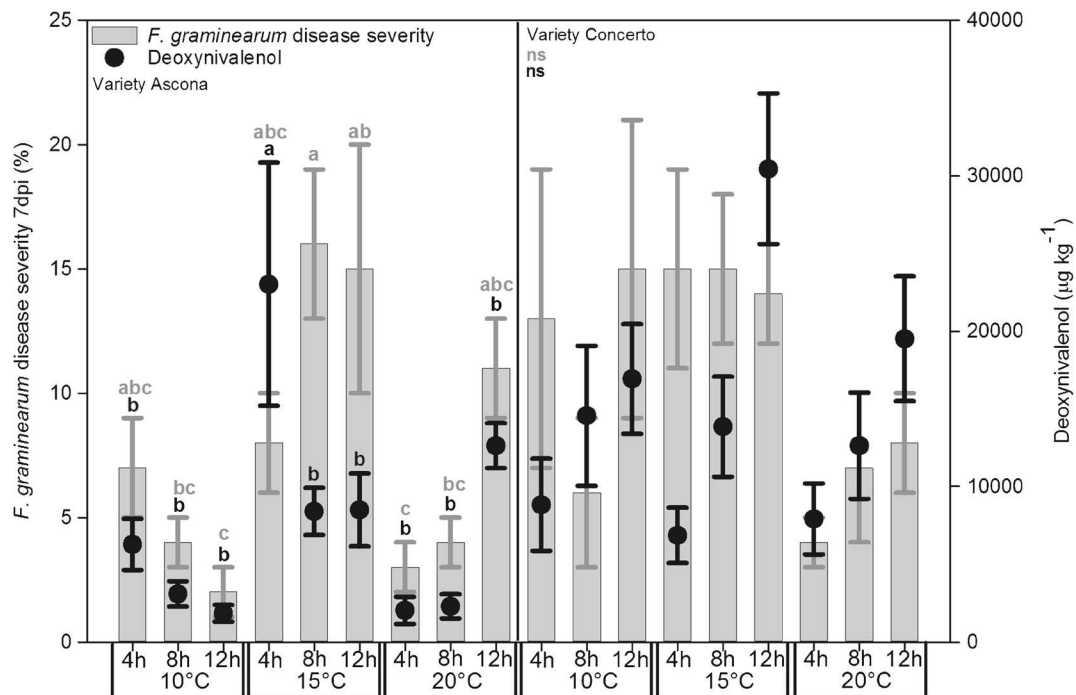


Fig. 2 Effect of temperature and humidity duration on *Fusarium graminearum* (FG) disease severity (%) based on the number of infected spikelets per spike after 7 days past inoculation (dpi) and deoxynivalenol (DON) content ($\mu\text{g kg}^{-1}$) in harvested grains of the barley varieties Ascona (left) and Concerto (right) in a climate

chamber. Pooled data from two experimental runs. $n = 54$ per variety. Grey and black letters represent significant differences for the FG disease severity and the DON content, respectively. Error bars represent the standard error of the mean, means with the same letters are not significantly different ($\alpha = 0.05$); ns = not significant

The lme revealed temperature and the interaction of temperature and humidity duration as significant factors on the DON content for variety Ascona (Table 1). Grains of plants inoculated at 15 °C / 4 h 99% RH resulted in the highest DON contamination and this treatment was significantly different ($p < 0.001$ – 0.038) from all other treatments (Fig. 2).

For the FG DNA amount, temperature was the only significant factor in the lme (Table 1). The 15 °C treatments resulted in a significantly higher DNA amount in the barley grains compared with the 10 °C ($p = 0.004$) and the 20 °C ($p = 0.002$) treatments (Fig. 3).

For the variety Concerto, only the temperature showed a significant effect on disease severity (Table 1). The grains of the plants inoculated during 15 °C revealed a significantly higher severity (14%) ($p = 0.013$) compared with grains inoculated during 20 °C (6%).

The humidity duration was the only significant factor in the lme for the FG DNA amount and the DON contamination (Table 1). The treatment 12 h

at 99% RH resulted in a significantly higher FG DNA amount ($p = 0.017$) and DON contamination ($p < 0.001$) compared with grains of the plants inoculated during 4 h at 99% RH (Fig. 4).

The highest FG DNA amount (484 copies / ng DNA extracted) and DON content ($23,014 \mu\text{g kg}^{-1}$) for the variety Ascona were detected in plants incubated at 15 °C and with 4 h 99% RH and for plants from the variety Concerto at 15 °C / 12 h 99% RH (530 copies / ng DNA extracted; $30,433 \mu\text{g kg}^{-1}$). For the variety Ascona, a significant correlation between disease severity and FG DNA amount ($r = 0.41$; $p = 0.002$) as well as for disease severity and DON contamination ($r = 0.57$; $p < 0.001$) was observed. The correlation of FG DNA amount and DON content was also significant ($r = 0.47$; $p < 0.001$). The same was observed for the variety Concerto, which revealed correlations between the disease severity and FG DNA amount ($r = 0.36$; $p = 0.009$) and with the DON content ($r = 0.45$; $p < 0.001$) as well as between the FG DNA amount and the DON content ($r = 0.42$; $p = 0.002$).

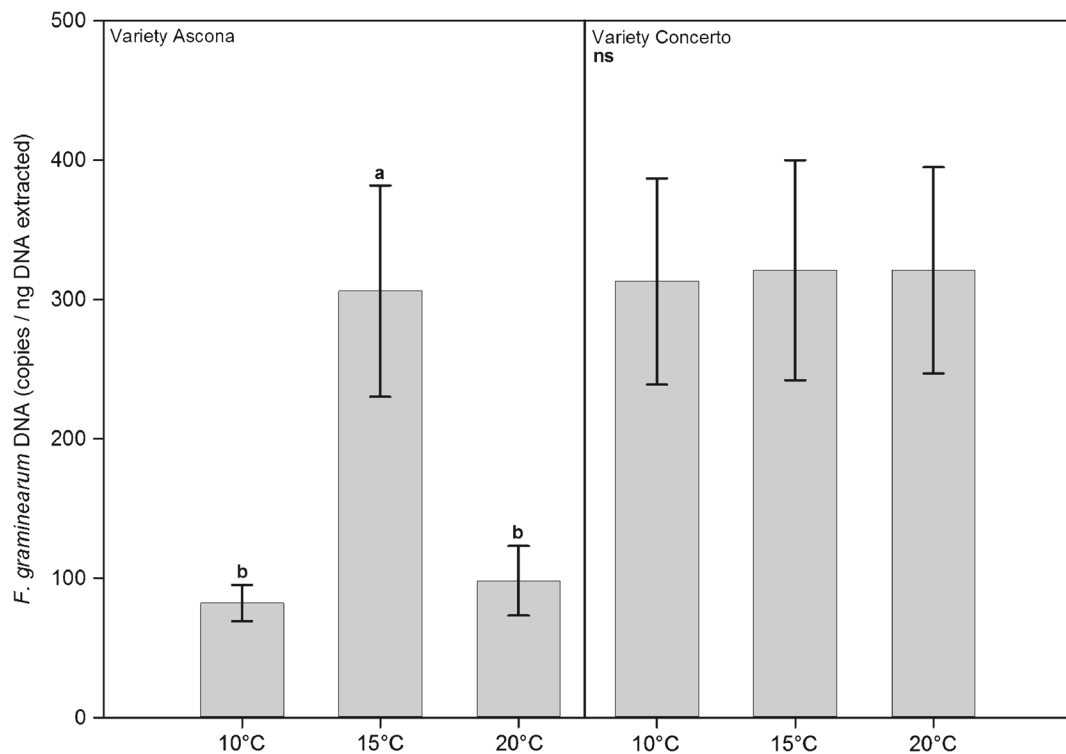


Fig. 3 Effect of temperature on *Fusarium graminearum* DNA amount (copies / ng DNA extracted) in grains of the barley varieties Ascona (left) and Concerto (right) in a climate chamber.

Pooled data from two experimental runs. $n = 54$ per variety. Error bars represent the standard error of the mean, means with the same letters are not significantly different ($\alpha = 0.05$); ns = not significant

Field experiment: Weather conditions and spore trapping

During the growth stages DC 25 to 51 (time of straw application until ear emergence), weather conditions in 2015 were characterised by a higher precipitation but a slightly lower mean RH compared with 2016 (Table 2). During DC 51 to 59 (ear emergence) a higher precipitation and RH was observed in 2015 compared with 2016. During anthesis (DC 61 to 69), less precipitation and higher temperature was observed in 2015 compared with 2016 (Table 2). After anthesis, more precipitation was observed in 2016 than in 2015. Generally, temperatures in 2016 were lower than in 2015.

In 2015, high precipitation (> 10 mm) and temperatures between 11 °C and 16 °C were recorded one week before anthesis, 1.2 mm at DC 61 (beginning of anthesis), no precipitation at DC 65 (full anthesis) and 20 mm at DC 69 (end of anthesis).

In 2016, no rain and temperatures between 9 °C and 16 °C were measured at DC 57/59 (end of ear emergence) and at DC 61 and DC 69 (Fig. 5). Temperatures above 15 °C were only measured during the first two days of DC

61. During DC 65 (anthesis half way) the highest amount of precipitation was observed from 12.05.16–14.05.16, with three days more than 20 mm (Fig. 5).

Due to sampling errors, no spore trap data were available in 2015. In 2016, the highest amount of FG colonies were detected at the end of anthesis, with on average 10 colonies per trap (15.05.16) (Fig. 5). On average, more FG colonies were detected 6 days before anthesis (5.4 colonies per trap per day) than during anthesis (3.4 colonies per trap per day) or three days after anthesis (3.2 colonies per trap per day). Apart from FG, *F. equiseti* was the second most occurring species detected in the spore traps, with 8 colonies during the entire sampling season. Other *Fusarium* species were only found once including *F. crookwellense*, *F. avenaceum*, *F. proliferatum*, *F. sporotrichioides* and *F. poae*.

Field experiment

The straw subsamples that were taken before the straw application and placed on PDA plates resulted in the presence of FG on the freshly inoculated and overwintered straw treatments, with an average incidence of 80% and

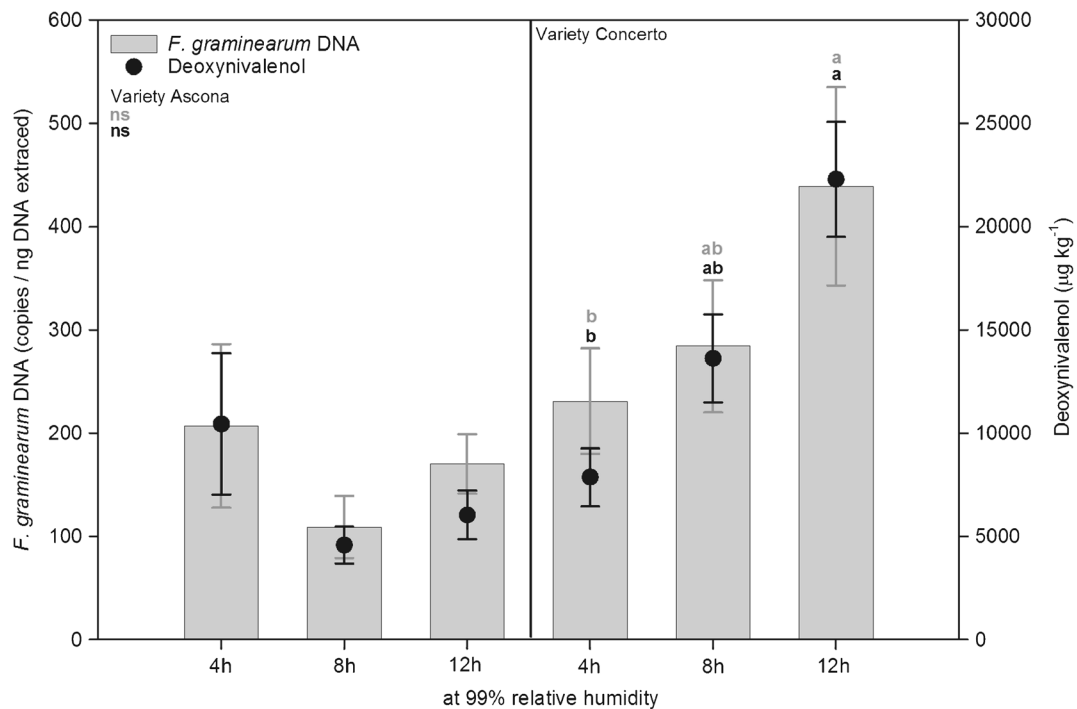


Fig. 4 Effect of duration at 99% relative humidity on *Fusarium graminearum* (FG) DNA amount (copies / ng DNA extracted) and deoxynivalenol (DON) content ($\mu\text{g kg}^{-1}$) in grains of the barley varieties Ascona (left) and Concerto (right) in a climate chamber. Pooled data from two experimental runs. $n = 54$ per variety. Grey

and black letters represent significant differences for the FG DNA amount and the DON content, respectively. Error bars represent the standard error of the mean, means with the same letters are not significantly different ($\alpha = 0.05$); ns = not significant

76%, respectively. No FG growth was observed on straw pieces of the control treatment (data not shown).

Visual assessment of the disease severity

The disease severity was significantly higher ($p < 0.001$) in 2016 compared with 2015 for both varieties, Meridian and Semper. In 2015, a significantly higher disease

severity was observed on plants of the variety Meridian from the freshly inoculated straw plots (2.8%) compared with the overwintered straw plots (0.8%; $p = 0.009$) and the control plots (1.3%, $p = 0.030$). In 2016, a higher disease severity was observed on plants from the overwintered straw plots (5.5%) which was significantly different from plants from the freshly inoculated plots (3.3%, $p = 0.024$). For variety Semper, no significant

Table 2 Mean relative humidity (%), temperature ($^{\circ}\text{C}$) and sum of precipitation (mm) during different barley growth stages (DC) measured in 2015 and 2016

Year	Period	DC*	Relative humidity (%)	Temperature ($^{\circ}\text{C}$)	Precipitation (mm)
2015	24.03. – 01.05.	25–51	68	9.6	204
2015	02.05. – 06.05.	51–59	84	15.0	69
2015	07.05. – 15.05.	61–69	68	15.8	24
2015	16.05. – 25.06.	71–92	73	16.1	135
2016	17.03. – 03.05.	25–51	75	8.5	110
2016	04.05–09.05.	51–59	60	13.4	0
2016	10.05. – 16.05.	61–69	79	12.1	91
2016	17.05. – 07.07.	71–92	78	16.5	265

*25: main stem and five tillers; 51: tip of ear just visible; 59: ear emergence complete; 61: beginning of anthesis; 69: anthesis complete; 71: kernel water ripe; 92: Grain hard, not dented by thumbnail; according to Zadoks et al. (1974)

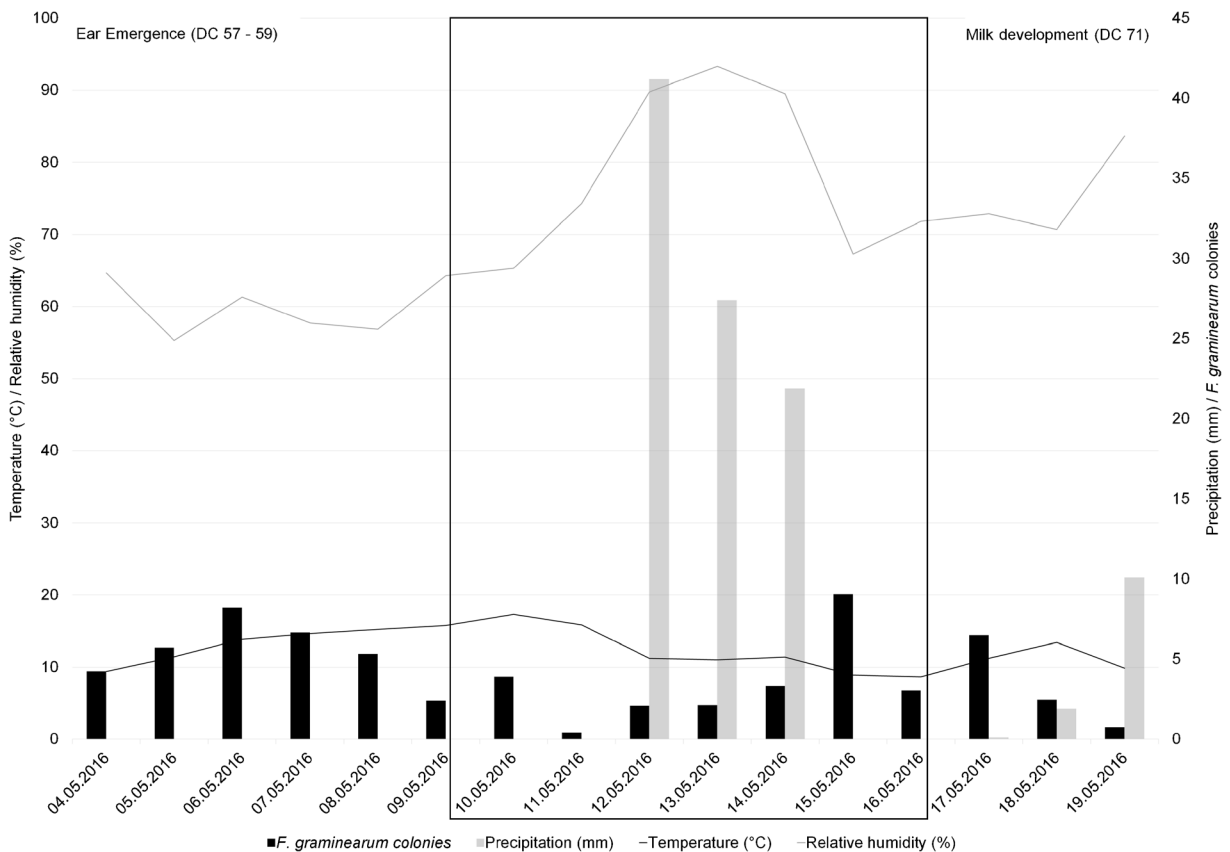


Fig. 5 Weather data and detected *Fusarium graminearum* colonies on agar plates containing the *Fusarium* selective substance pentachloronitrobenzene between 04.05.2016 (DC 57) and 19.05.2016 (DC 71) in a field experiment. Weather data are represented on a daily basis by the sum of precipitation (mm),

the mean temperature (°C) and the relative humidity (%). The anthesis period of barley (10.05.2016–16.05.2016) is indicated by the black frame. No spore trap data are available for the year 2015

differences in disease severity were observed between the three straw inoculation treatments in any of the two years (data not shown).

Fusarium graminearum incidence

In both years, 2015 and 2016, from all identified *Fusarium* species in the grains, FG was the dominant species, with ratios of 100% and 97%, respectively. In addition, in 2015 only *F. poae* (0.5%) and in 2016, 2% *F. avenaceum* and 1% *F. poae* were identified.

Similar to the disease severity, a strong year effect was found for the FG incidence in grains which was for the variety Meridian significantly ($p = 0.033$) lower in 2015 (13%) compared with 2016 (23%). The same was observed for the variety Semper ($p < 0.001$) with 4% FG in 2015 and 27% in 2016.

In 2015, a significantly higher FG incidence was observed in grains from the overwintered straw treatment compared with the control treatment ($p = 0.041$) for the variety Meridian (Fig. 6). The same trend ($p = 0.064$) was observed for variety Semper. No significant differences between the three straw treatments were observed in 2016 for both varieties (Fig. 6).

Deoxynivalenol content

The maximum level for unprocessed barley for human consumption of $1250 \mu\text{g kg}^{-1}$ was exceeded four times out of the twelve plots in 2015 and never in 2016. Only in 2015, no DON was detected in grains of the control plots of both varieties. For Meridian, the average DON contamination was nearly equal in both years with $451 \mu\text{g kg}^{-1}$ in 2015 and $441 \mu\text{g kg}^{-1}$ in 2016. Grains from the variety Semper showed a higher mean

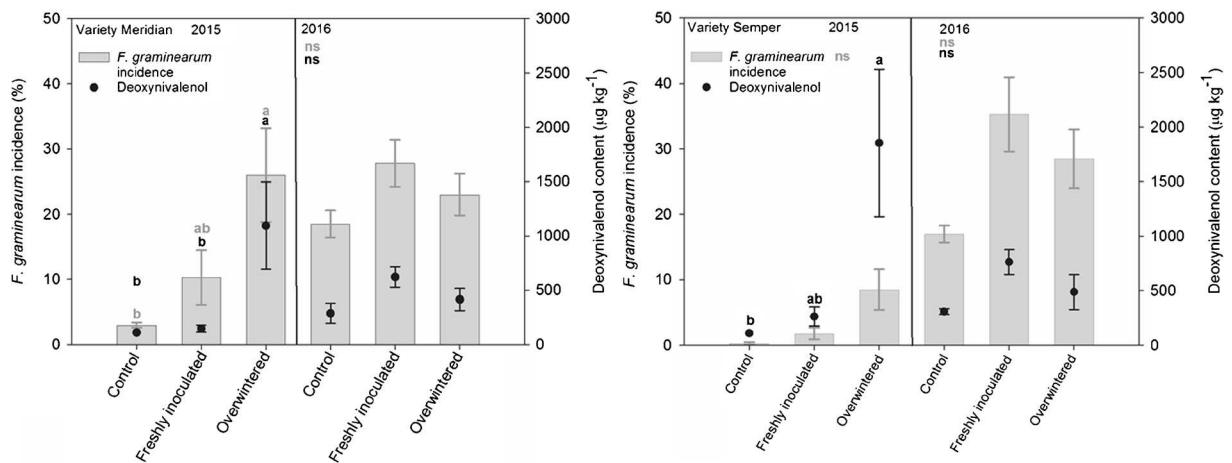


Fig. 6 Effect of the straw inoculation treatments on *Fusarium graminearum* (FG) incidence (%) and on the deoxynivalenol (DON) content ($\mu\text{g kg}^{-1}$) in grains of the barley varieties Meridian (left) and Semper (right) in field experiments, conducted in 2015 and 2016. The statistical analysis was conducted for each year and

each variety separately (each $n = 12$). Grey and black letters represent significant differences for the FG incidence and the DON content, respectively. Error bars represent the standard error of the mean, means with the same letters are not significantly different according to a Tukey test at $\alpha = 0.05$; ns = not significant

contamination in 2015 ($743 \mu\text{g kg}^{-1}$) compared with 2016 ($520 \mu\text{g kg}^{-1}$).

A significant effect of the straw inoculation treatments on the DON content was observed in 2015, where grains of the variety Meridian from plots with overwintered straw contained more DON compared with those from the control plots and the freshly inoculated plots ($p = 0.024$, $p = 0.042$, respectively) (Fig. 6). For variety Semper, this effect was only observed for the control treatment ($p = 0.028$) (Fig. 6). No significant differences between the three straw treatments were observed in 2016 for both varieties.

A significant correlation between FG incidence and DON contamination was observed in 2015 ($r = 0.58$; $p = 0.003$) and 2016 ($r = 0.59$; $p = 0.002$). In contrast, no significant correlation was detected between the disease severity and the DON contamination in any of the two years. The disease severity correlated only in 2015 with the FG incidence, ($r = 0.49$; $p < 0.001$).

Discussion

The climate chamber experiments demonstrated that overall, a FG infection of barley is possible under all tested climatic conditions. However, in particular for the variety Ascona, incubation temperatures of 15°C were shown to be the most favourable in terms of severity and DON contamination. This finding is in contrast to the results of other authors that FG is generally associated

with warmer regions of the world and was shown to cause higher infections in wheat at temperatures $\geq 20^\circ\text{C}$ (Osborne and Stein 2007; Xu et al. 2007). Experiments under controlled conditions showed that a higher FG infection frequency (Rossi et al. 2001) or FG DNA content (Brennan et al. 2005) was measured during inoculation at temperatures $\geq 20^\circ\text{C}$ compared with cooler temperatures. We assume that the cooler conditions during the earlier anthesis of barley compared with wheat are more favourable for an FG infection. For the variety Ascona, we observed a higher disease severity and DON contamination in the 10°C treatments after 4 h at 99% RH, compared with the 8 h and 12 h treatments. It is possible that the increased temperature of 20°C after 4 h of high humidity promoted the growth rate of already germinated spores (Wagacha et al. 2012; Beyer et al. 2004). For the variety Concerto, the humidity duration had a bigger influence on FG DNA amount and DON contamination and resulted in higher levels following a prolonged humidity duration. In general, the variety Concerto, showed a lower resistance and higher DON contamination, which resulted in less distinct differences between the treatments.

The longer humidity duration probably enhanced the germination rate and time frame to infect the plant which led to more DON contamination, as shown under controlled conditions in wheat (Xu et al. 2007; Martínez et al. 2012; Beyer et al. 2005). Hence, at higher temperatures, rainy periods or long periods of mist during barley anthesis can increase the risk of FG infections.

Several studies have shown that FG overwinters on crop residues, which in turn serve as inoculum in the subsequent growing season (Dill-Macky and Jones 2000). Thus, the higher FG incidence in 2015 in the overwintered treatment compared with the freshly inoculated treatment occurred most probably due to a faster colonisation and development of FG on the overwintered straw residues. This effect was not observed in 2016, probably due to the generally more favourable infection conditions (higher precipitation and humidity during anthesis), which attenuated the differences between the treatments.

In 2015, the year with less favourable infection conditions, differences between the varieties were observed, which might be due to phenological differences. Meridian is shorter than Semper, so the ears are closer to the ground which facilitates an infection through ejected ascospores from the soil surface as it was observed in wheat (Maji and Imolehin 2002).

The fact that FG infection in 2015 occurred might be due to the precipitation before anthesis, washing spores into the flag leaf containing the barley ear with the exposed anthers, which in fact was observed by other authors under field and controlled environment conditions (McCallum and Tekauz 2002; Osborne and Stein 2007). Furthermore, it is possible that barley possesses a prolonged period of susceptibility, since high FG incidences and DON contaminations were observed in a spring barley field experiment when inoculations were conducted as early as DC 51, DC 56 or DC 65 (L. Stauber personal communication). This implies a further difference with wheat, where anthesis is known to be the main susceptible period (Osborne and Stein 2007). However, wheat forecasting models usually include a pre-anthesis period to predict the DON contamination more reliable. Still, it must be noted that spring barley develops rapidly and that anthesis mostly occurs during head emergence (DC 51–59). Thus, a clear differentiation of the growth stages is difficult as recently shown by Alqudah and Schnurbusch (2017).

Despite the more favourable infection conditions due to higher precipitation during anthesis (Hooker et al. 2002; Brustolin et al. 2013), only few FG colonies were noted during anthesis compared with the occurrence before anthesis. This suggests that potentially ejected ascospores were washed down by the higher precipitation or that water covering the perithecia inhibited ejection as assumed by Paulitz (1996). Moreover, during anthesis in 2016, temperatures were unusually cool. The

ejection of ascospores is not only favoured by high humidity but takes place in a range between 10 °C and 30 °C with an optimum near 16 °C (Doohan et al. 2003; Sutton 1982; Paulitz 1996). Indeed, the Swiss forecasting system FusaProg for FHB and DON in wheat calculates main infection and sporulation days with temperatures above 15 °C (Musa et al. 2007). Hence, the temperatures below 15 °C were possibly not favourable for ascospore ejection during anthesis. However, because spore trap data were only available for 2016, the results need to be interpreted with caution.

The higher precipitation occurring after anthesis in 2016 not only might have enhanced the spread of the fungus within the plant but also might have triggered the production of new conidia. The splash dispersal of conidia and ascospores after anthesis under wet conditions was also observed in field experiments with wheat (Paul et al. 2004; Manstretta et al. 2015). Furthermore, studies under controlled and field conditions showed that cleistogamous barley varieties were resistant during anthesis but became susceptible afterwards (Yoshida et al. 2007; Yoshida et al. 2008). The barley varieties in our field experiment are both closed flowering and thus, might behaved similarly. To test this hypothesis, further climate chamber experiments with different barley varieties inoculated at different growth stages will be necessary.

Based on the high disease severity in the field and elevated FG incidences in harvested grains in 2016, we expected a higher DON contamination. However, the measured DON values were nearly equal and in some samples even slightly lower than in 2015. It must be noted that the higher DON content in 2015 was mainly a result of the high contamination of the overwintered straw plots. We assume that the high precipitation at the end of the growing season caused leaching of the water soluble DON, as was observed in field studies artificially spray inoculated with FG in the USA and in Switzerland (Gautam and Dill-Macky 2012; Schenzel et al. 2012).

The close correlations between disease severity, FG DNA amount and DON in the climate chamber demonstrate that a rough estimation of the DON content in barley based on visual assessments is possible under controlled conditions. Under field conditions, however, the disease severity showed only a close correlation with the FG incidences. Only 100 ears of the barley plot were examined for the disease rating, whilst the data of seed health test and DON were based on grains from the entire plot, which provided a higher representative sample. Since in the field, many other factors can influence the appearance

of symptoms, we assume that an estimation of the DON content is not possible by visual assessment. Nevertheless, the FG incidences and the DON contents were closely related, which showed that FG was the main DON producer, as observed in other studies with several cereal types (Hietaniemi et al. 2016; Lindblad et al. 2013).

Conclusion and outlook

Our results indicate that the optimum temperature for FG infection of barley is 15 °C, which is different from the warmer infection requirements known for FG in wheat. The duration of high humidity was much more important for the variety Concerto, which indicates that infection conditions can be variable for different varieties. This could also be an effect of the different purposes (feed or malt) and thus distinct grain constitution of the two varieties. Hence, we assume that the definition of weather conditions for barley are more challenging than those in wheat. For the development of a forecasting system for FHB and DON in barley, the potentially longer time frame of susceptibility should be taken under consideration and needs to be verified by more detailed climate chamber and field experiments including several barley varieties. Furthermore, the weather conditions after anthesis until harvest can affect the DON content in the grains and should be investigated in multi-site and multi-year field experiments. Also studies comparing spring and winter barley as well as selected spring and winter wheat varieties should be conducted to observe potential phenological differences such as row type (in case of barley), flowering type and anthesis period on infection with FG and DON contamination. The processing (sterilisation, mixing and application) of the inoculated straw, as well as the weighing and distribution in the field resulted in a large volume. Hence, for future studies we recommend to use maize stalks as inoculum source.

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Compliance with ethical standards

Conflict of interest No potential conflict of interest was reported by the authors.

Human participants and animal studies This research did not involve human participants or any animal experimentation.

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